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14. ABSTRACT <p>C-peptide is the segment connecting insulin A and B chains. It is generated in pancreatic beta cells as the natural product of pro-insulin cleavage. For a long time, it was considered biologically important only for favoring pro-insulin folding within the secretory granules of the beta cells. Consistently with this view, the standard of care for diabetic, and especially T1D patients is solely insulin-replacement therapy; C-peptide is not administrated. However, recent studies have challenged this view. It has been offered increasing evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for diabetic patients who suffer from micro-vascular complications. How exactly C-peptide achieves these intracellular effects, however, is still unknown. One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization.</p>					
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**University of Pittsburgh
W81XWH-10-1-1055
Annual Report (09/28/2012 – 09/27/2013)
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INTRODUCTION/ORIGINAL STATEMENT OF WORK

Diabetes mellitus is a life-threatening disease that places children (type 1) and adults (type 2) at risk of complications of blindness, kidney damage and heart disease. Diabetes afflicts 16 million Americans, with more than 800,000 new cases diagnosed each year. African, Hispanic, Native and Asian Americans are particularly susceptible to its most severe complications. Costs associated with diabetes are estimated to reach \$132 billion/year. Significantly, the number of diabetes cases in the United States mirrors national rates. Type 1 diabetes (T1D) patients lack physiological levels of insulin in their bloodstream due to the autoimmune destruction of the insulin producing pancreatic beta cells. Type 2 diabetes (T2D) patients are, instead, afflicted by a heterogeneous set of sub-syndromes characterized by peripheral insulin resistance with or without production insufficiency. Both T1D and T2D patients are at increased risk for damages of both micro- and macro-vascular tissues, which eventually bring to the well known, tragic, diabetic complications.

C-peptide is the segment connecting insulin A and B chains. It is generated in pancreatic beta cells as the natural product of pro-insulin cleavage. For a long time, it was considered biologically important only for favoring pro-insulin folding within the secretory granules of the beta cells. Consistently with this view, the standard of care for diabetic, and especially T1D patients is solely insulin-replacement therapy; C-peptide is not administrated. However, recent studies have challenged this view. It has been offered increasing evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for diabetic patients who suffer from micro-vascular complications. How exactly C-peptide achieves these intracellular effects, however, is still unknown.

In preliminary results, we have demonstrated that C-peptide reduces secretion of inflammatory cytokines from endothelial cells in a model of hyperglycemia-induced vascular injury by reducing activation of the nuclear factor (NF)- κ B pathway (1). We found a similar anti-inflammatory activity of C-peptide in vascular smooth muscle cells (2). For the full-length, native, C-peptide, we found that, upon internalization from the cell surface, C-peptide quickly traffics to early endosomes and later proceeds to lysosomes for degradation (3). Trafficking of C-peptide to early endosomes is likely to account for its anti-inflammatory effects in vascular endothelial and smooth muscle cells. Based on these findings, it is hypothesized that C-peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes (4). One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization (5). In this project, we will set a number of experiments specifically designed to isolate the CPR and characterize its intracellular signaling activity, with the following specific aims:

1. To isolate the C-Peptide Receptor (CPR). We have designed and synthesized a set of biotinylated C-peptides including wild type and two mutants previously shown to not bind cellular surface membrane. These peptides will be allowed to internalize into endothelial and smooth muscle cells. Endosomes containing the biotinylated C-peptides will be isolated by cell fractionation, solubilized, and the C-peptide/CPR complexes isolated. Proteins of the wild type but not the mutant C-peptide/receptor complexes will be sequenced.

2. To identify endosomes as the subcellular site of C-peptide/CPR signaling. Endosomes are likely candidate sites for intracellular signaling by the C-peptide/CPR complex. In this aim, we will block C-peptide internalization at different stages, by using pertussis toxin and endosomal Rab5 trafficking mutants, to determine at which station along its endocytic route C-peptide activates its intracellular signaling pathway.

3. To investigate the anti-inflammatory effect of C-peptide on high glucose-induced vascular dysfunction *in vivo*. We have definite proof that C-peptide displays a powerful anti-inflammatory effect on endothelial cells *in vitro*. It is important to investigate whether this anti-inflammatory activity of C-peptide is also observed *in vivo*. To this aim, we will inject C-peptide in a mouse model of diabetes-induced vascular disease and study the effect on adhesion molecule expression and macrophage accumulation particularly in the aortic segment.

Our first quarterly scientific progress report for the third year of our project (09/28/12 – 12/27/12) described the following:

With regards to Aim 1.

Our attempts to cross-link the C-peptide sequence to a potential receptor have finally yielded convincing evidence that C-peptide is interacting with a cellular protein. After treating HEK293 cells (a Human embryonic kidney cell line) with our modified cross-linkable C-peptide for 1 hour followed by UV-activation of the cross-linking process, we have found a biotin and hemagglutinin positive band with an apparent molecular weight of 75 kd . This cross-linked molecule was recovered from the intact-cell supernate after incubation with C-peptide and was visualized by western blotting. Figure 1.

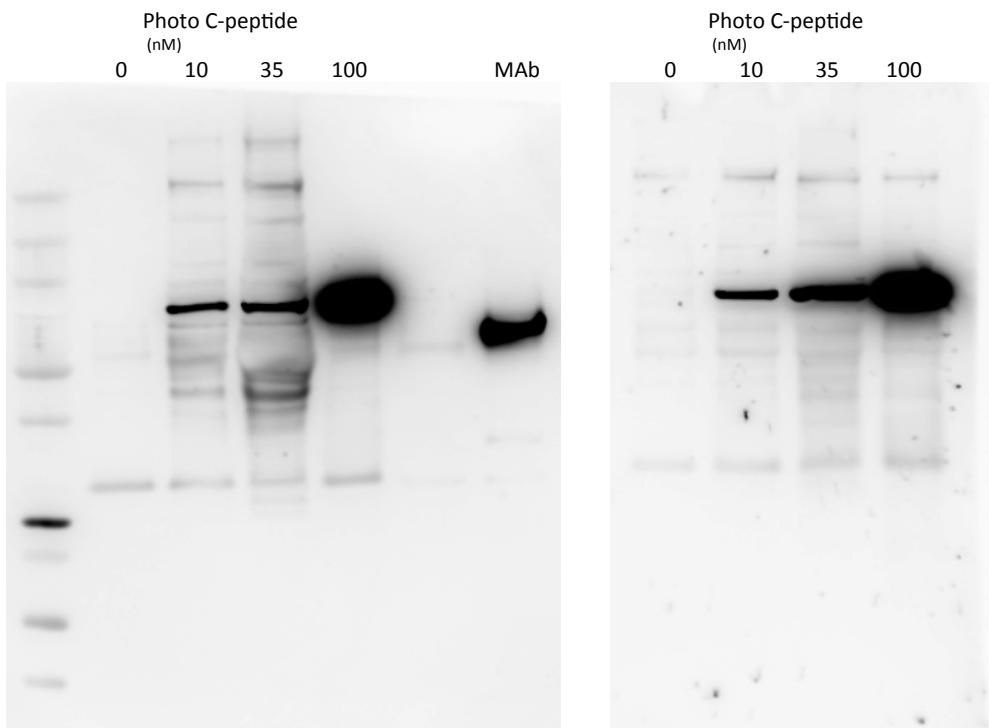


Figure 1. Modified C-peptide is cross-linked to a 70 kd extracellular protein.

7×10^5 HEK293 cells grown in serum free media were treated with three concentrations of the modified C-peptide for 1 hour. Cross-linking was activated by UV light exposure and the cross-linked molecules were captured by affinity chromatography via the biotin or HA epitopes of the modified C-peptide. The captured proteins were eluted with SDS sample loading buffer, separated by gel electrophoresis and revealed by western blotting and biotin detection. In the left panel the experiment was conducted in a binding buffer used in many reported C-peptide binding studies and in are earlier work. The right panel used Hank's Balanced Salt Solution (HBSS) instead. The two right lanes of the left panel are cross-linking controls that consist of a monoclonal C-peptide antibody and the modified C-peptide without or with UV light exposure. The cross-linked molecule has an apparent size of 75 kd

We found equivalent results when the western blots were probed for biotin, the HA epitope or using C-peptide antibody. Figure 2. This was expected because our modified, crosslinkable C-peptide has all of these epitopes. Other bands, not observed in all of the western blots, are most likely artifacts related to the individual reagents used in each detection method. We had previously reported a similar result from soluble lysates of rat macrophages, but had difficulty reproducing the result and also in expanding the production of cells sufficiently to support further studies. We now suspect that the large quantity of protein in the cell lysates was interfering with the affinity capture of biotin by non-specific adsorption to the affinity matrix. In the current experiments, this has been overcome by the serum free culture of the HEK293 cells and the finding that the cross-linked molecule is relatively more abundant in the extracellular solution. We expect that this insight will help us to modified our methods to be able to study other cells lines, including adherent cells and ones grown in the presence of serum.

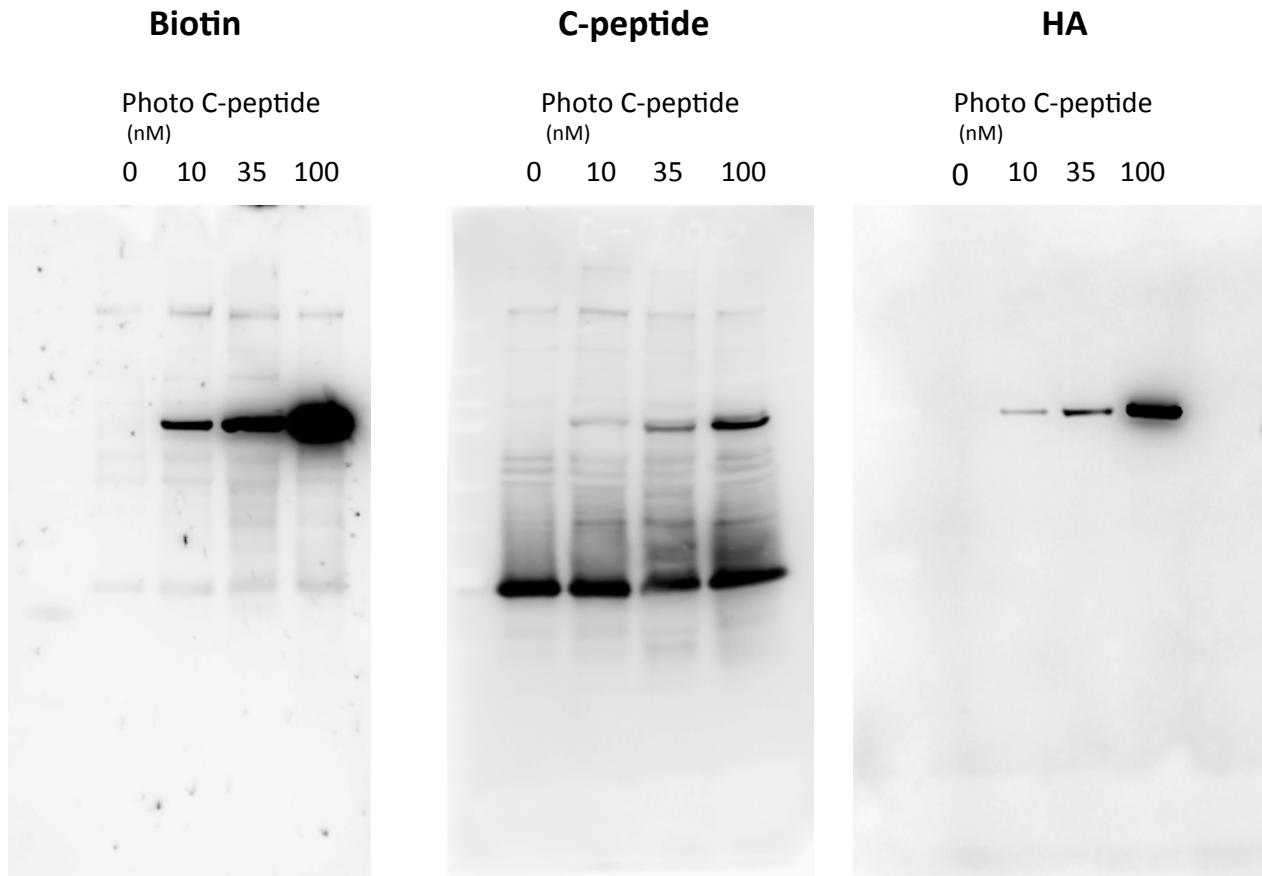


Figure 2. The cross-linked C-peptide molecules are detectable with three different reagents.

The left panel is the same as in Figure 1, and the other panels use additional aliquots from that experiment. The left and right panels are developed with direct HRP-labelled reagents for biotin or HA. The center panel uses a secondary reagent to detect a C-peptide monoclonal antibody, but also shows a non-specific interaction with a smaller abundant protein that is not seen in the left and right panels.

The presence of the cross-linked molecule in the extracellular solution does not seem to be a consequence of the incubation in HBSS solution because we have found similar results when we used culture media instead. However, in this case, it was necessary to desalt the collected samples before cross-linking to remove small molecules especially free amino acids that would compete in the cross-linking reaction. Figure 3.

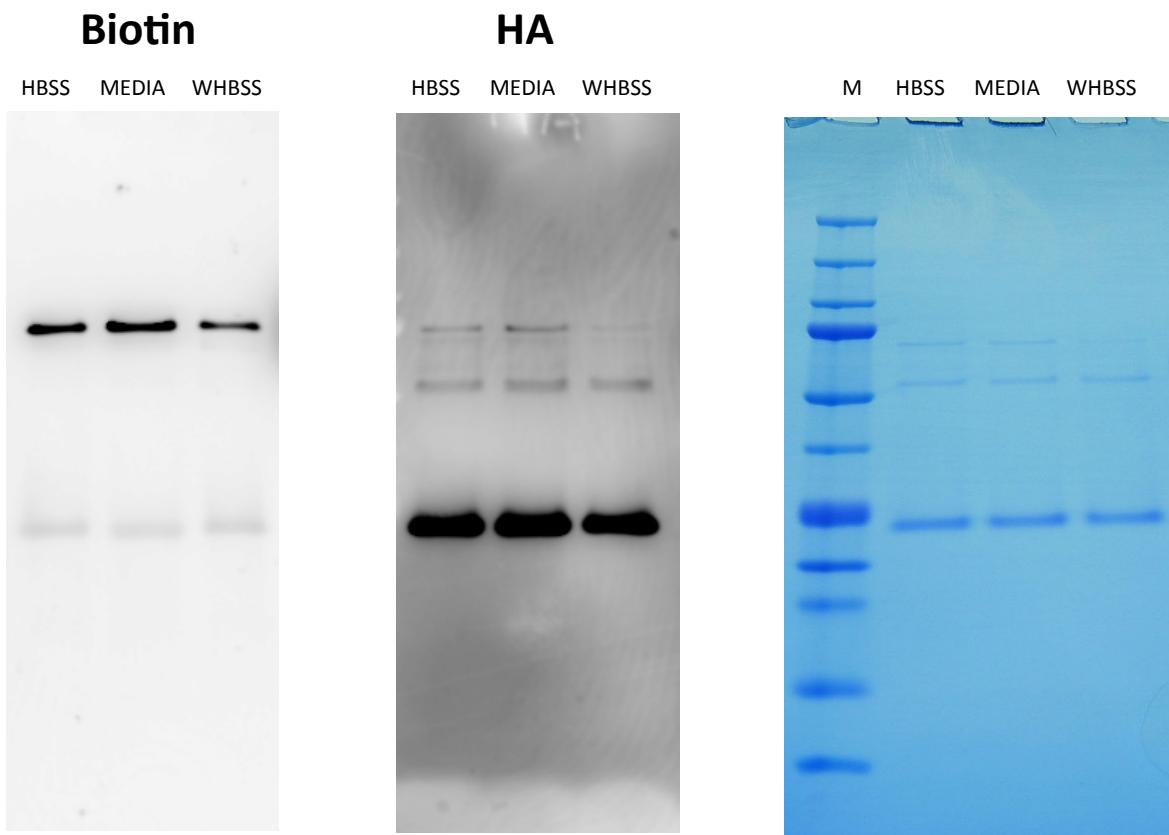


Figure 3. Culture media does not prevent the appearance of the C-peptide interacting protein in the extracellular solution.

HEK293 cells were collected by centrifugation and resuspended with C-peptide in HBSS or in fresh culture media. In the lanes labeled WHBSS, the cell pellet was briefly washed with HBSS and then resuspended in HBSS containing C-Peptide. After 1 hour, the cells were centrifuged, the supernatant was collected and each sample was desalting so that free amino acids in the media could be removed so as to not interfere with cross-linking. There is no reduction in the signals for either biotin or HA when comparing culture media to HBSS. However the single wash with HBSS slightly reduced the signal. The right panel is a gel stained with Coomassie Blue and does not reveal any detectable signal corresponding to the position of the cross-linked molecules. The smaller non-specific band seen in Figure 2 is visible in the biotin and HA blots in this experiment but also appear to be sufficiently abundant in the Coomassie Blue stained gel to explain the non-specific interaction of the detection reagents

We have also demonstrated that this cross-link dependent signal is specific for the native C-peptide sequence since it is reduced when the incubation with the modified C-peptide is done in the presence of a 13 fold excess of native C-peptide. Figure 4.

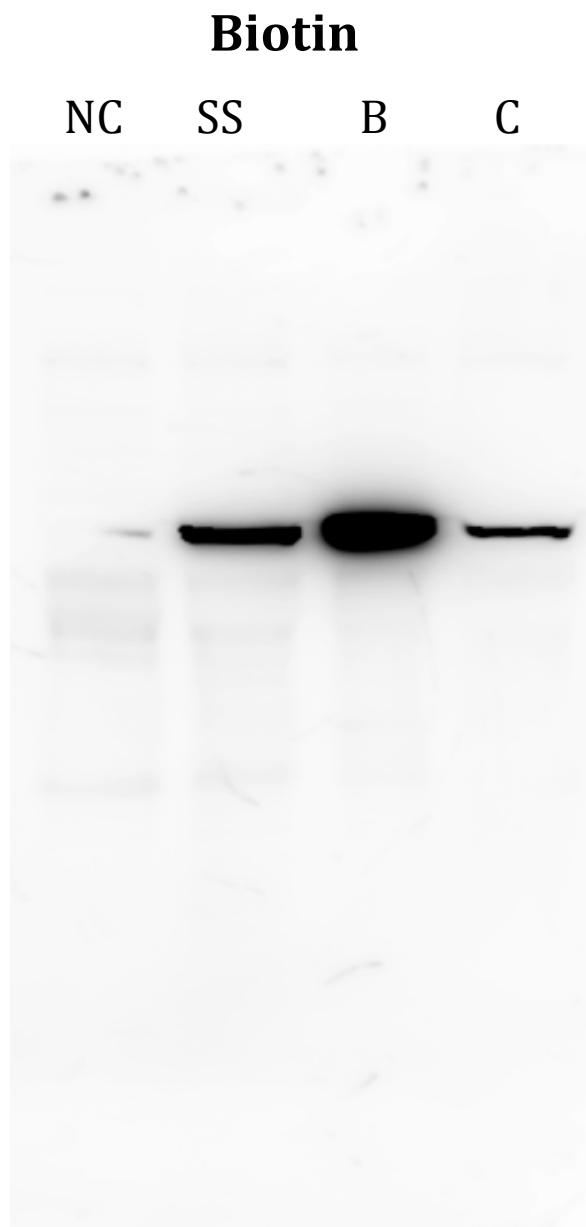


Figure 4. Native C-peptide competes with the modified C-peptide during the interaction that is stabilized by cross-linking.

In this experiment the lanes labeled NC, SS, and C, the affinity capture utilized beads able to bind the HA epitope. NC, no UV crosslinking; SS, standard extracellular soluble fraction prepared as in Figure 1, and 2 with 75 nM modified C-peptide; C, like SS except that there was a 13 fold molar excess of native C-peptide. We also compared the efficiency of the affinity capture using the HA epitope (SS) versus biotin (B) to guide our future efforts to purify the cross-linked molecule.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

We are now preparing a sufficient quantity of the cross-linked molecule to be identified by mass spectroscopy. Our future work will focus on confirming the mass spectroscopy identification, exploring the biological and physiological significance of the identified protein, and investigating the presence and function of the identified protein in other cell lines that have shown physiological responses to C-peptide.

Our second quarterly scientific progress report for the third year of our project (12/28/12 – 03/27/13) described the following:

We have previously found that after treatment of HEK293 cells with a cross-linkable C-peptide there is an extracellular release of an unknown protein cross-linked to C-peptide. In this quarter we have affinity purified sufficient quantities of our unknown C-peptide binding molecule to identify it by mass spectroscopy. The results indicated that our 75 kD band is the 70 kD protein, HSP70A1A/B. The 75 kD band is compatible with the combined size of HSP70A1A/B cross-linked to C-peptide and the statistical certainty of this identification is very significant, $\log(e) -271$. The only other potential candidate was larger and had a molecular weight of 73.6 kd without taking into consideration the additional mass of the C-peptide. It is likely that this was a non-cross-linked contaminating protein migrating at its native size in the gel. However, we also observed a much stronger coomassie blue staining band at 70 kD in our preparative gel that was also found to be HSP70A1A/B by mass spectroscopy.

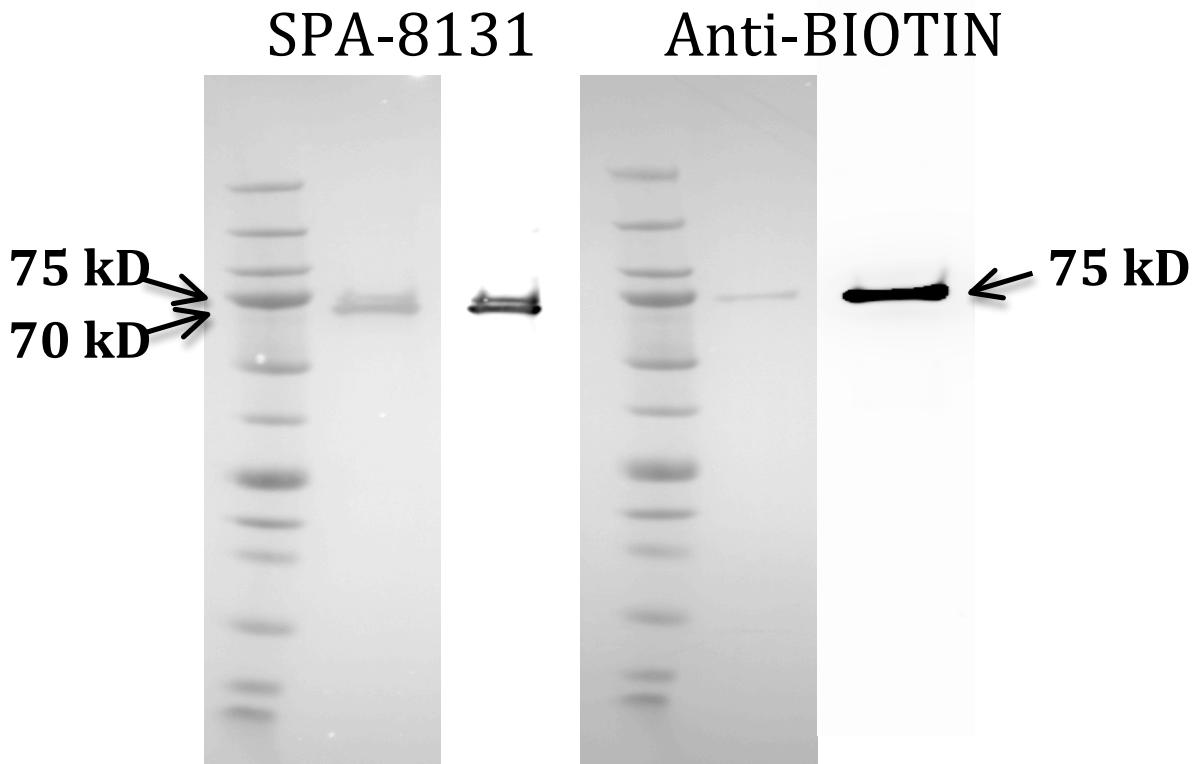


Figure 1. Confirmation of mass spectroscopy results with HSP70 antibody. HEK293 cells were treated with 50 nM C-peptide modified to include both biotin and the hemagglutinin epitope at the amino end and a photo-linkable leucine at position 21 of the sequence. After 40 minutes at 4°C the cells were UV irradiated for 20 minutes. Then after centrifugation, the supernatant was filtered and the C-peptide interacting products were affinity purified with anti-HA agarose. After SDS gel separation, western blots were prepared and analyzed with HSP70 antibody (SPA-8131, ENZO Life Sciences) or with anti-biotin antibody. The white columns show the chemiluminescent detection of the antibodies and the darker panels are photos of the membranes showing the size of the visible reaction by-products compared with the colored molecular weight markers at the left side of the images.

We were concerned that our identification might be compromised by this abundant smaller band trailing into the larger 75 kD region of our gel. This issue was resolved with the confirmation obtained with HSP70 antibody and the mechanistic properties of HSP70 peptide-binding.

In Figure 1, we show western blots of our C-peptide cross-linked samples developed with HSP70 antibody and with anti-biotin antibody. The exposed western membranes contained sufficient by-products of the luminescent reactions that an accurate comparison could be made with the colored molecular weight markers with visible light alone to unequivocally determine that both the 75 kD and the more abundant 70 kD gel bands reacted with the HSP70 antibody. These results indicate that the binding of C-peptide by HSP70A1A/B was strong enough to allow the affinity purification of HSP70 with

biotinylated C-peptide without cross-linking. This is possible because a lid structure of HSP70 can cover the peptide-binding domain to strongly anchor peptides bound to the peptide-binding domain of HSP70.

The binding properties of many HSP70-bound peptides suggest that amino sequences that bind to HSP70 may bind regardless of whether they are made of D or L amino acids or even if the sequence is reversed. Although these characteristics are unlike those of known stereo-specific hormone receptors, an early publication reports that the prevention by C-peptide treatment of vascular and neural dysfunctions of diabetes is not mediated by stereospecific receptors or binding sites (Ido et al., *Science* 277:563,1997). This conclusion was based in part on the nearly equal preventative effects of native C-peptide, non-native-D amino acid C-peptide, and of reverse-sequence C-peptide that would depend on peptide-binding characteristics like those of HSP70. Thus, it is reasonable to consider HSP70 as a candidate for a C-peptide “receptor” with physiological significance.

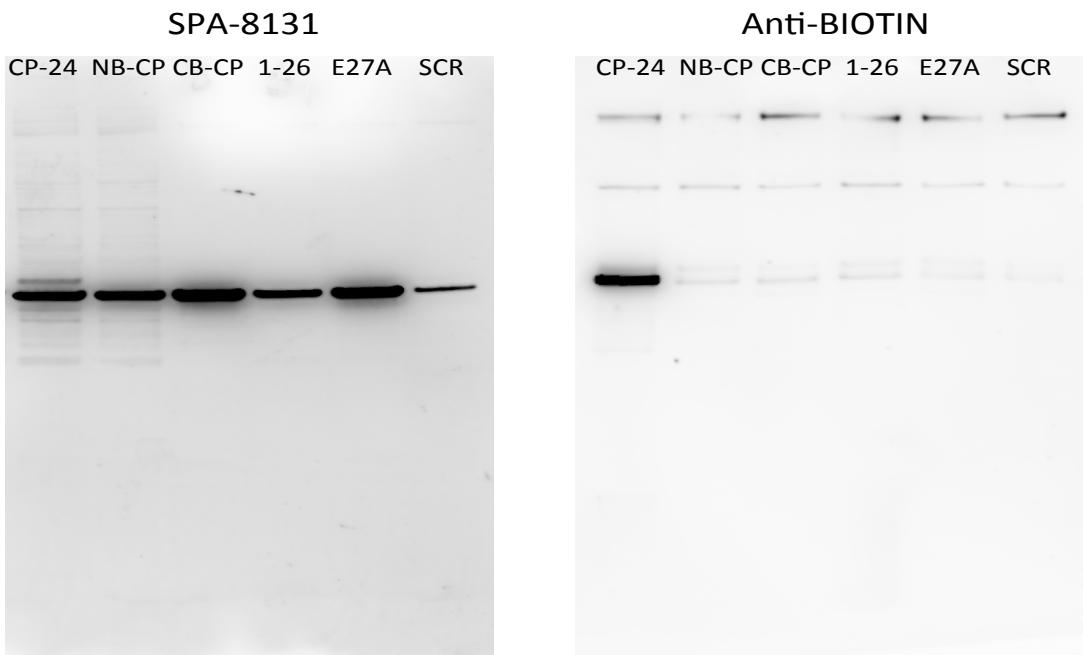


Figure 2. Capture of HSP70 with various modified C-peptides. Several biotinylated peptides related to C-peptide were used to affinity capture HSP70 as in Figure 1. CP-24 contains biotin and the HA antigen and a photo-linkable leucine at position 24. Only the CP-24 sample was UV treated as all the other peptides were just biotinylated. All these other peptides have biotin-KAAA added at the N-terminus, except CB-CP that has AAAK-biotin added at the C-terminus. NB-CP, N-terminal-biotin C-peptide; CB-CP, C-terminal-biotin C-peptide; 1-26, the first 26 amino acids of C-peptide; E27A, C-peptide with amino acid 27 changed to A; and SCR, a scrambled C-peptide sequence: DQEVELGAPQSAGLGGSQLEPQQLGLVLGE. The HSP70 antibody (SPA-8131) detects the 70 kD band as in Figure 1. The 75 kD band representing the cross-linked C-peptide is present as a less intense band in the CP-24 lane with HSP70 antibody and is also strongly biotin positive. Without the covalent link to HSP70, the other C-peptides are too small to be resolved and detected in these gels. The very weak biotin positive bands are endogenously biotinylated proteins that appear because avidin agarose was used instead of the HA agarose as in Figure 1.

We have begun to study the interaction of HSP70 and modified C-peptide sequences. Based on the non-cross-linked capture by C-peptide, we found that several different biotinylated C-peptide-related molecules bind to HSP70 as revealed by biotin-captured extracellular HSP70 (Figure 2). C-peptide biotinylated at the carboxyl-terminus was more effective than amino-terminal labeled C-peptide; When the carboxy-terminal pentapeptide was deleted there was only a small reduction of the captured HSP70; And when the glutamate at position 27, the first amino acid of the pentapeptide, was changed to alanine, there was no difference in the captured HSP70. These results are compatible with binding of the middle portion of C-peptide by HSP70A1A/B just as with the modified peptides used in the C-peptide functional study referenced above.

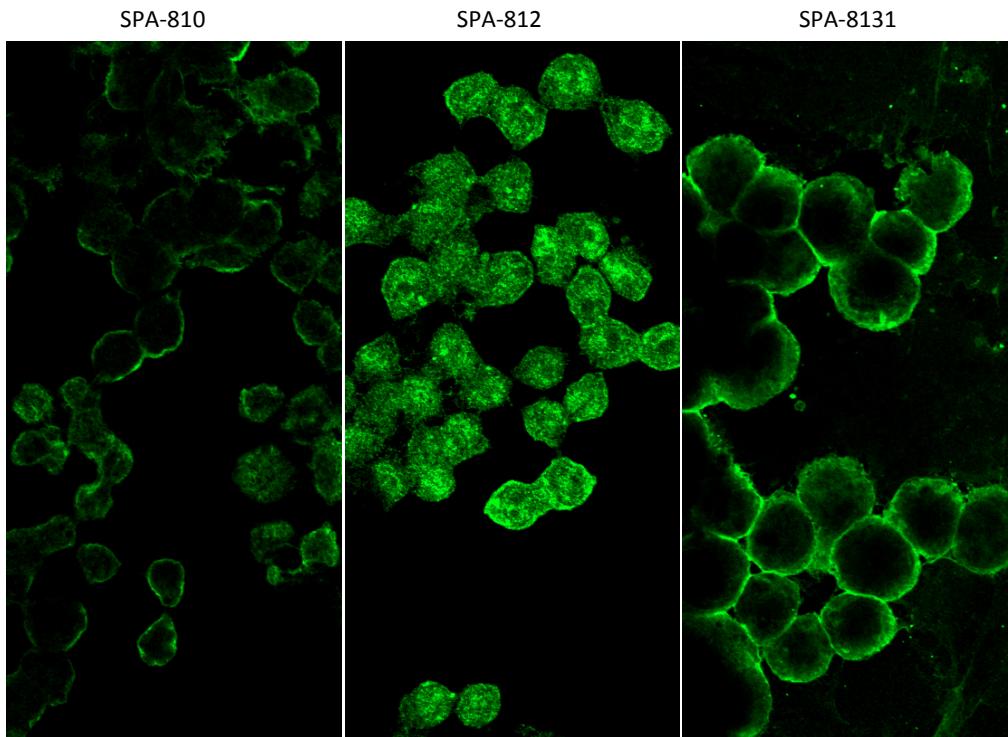


Figure 3. Confocal microscopy of HEK293 cells stained with HSP70 antibody. HEK293 cells were placed on slides, dried, fixed, and stained with HSP70 antibodies SPA-810, SPA-812, and SPA-8131 (Enzo Life Sciences).

We have also observed that the HSP70 monoclonal antibodies SPA-810 and SPA-8131 show that HSP70 is near or may be integrated into the plasma membrane of the HEK293 cells (Figure 3). However, the polyclonal antibody SPA-812 reveals cytoplasmic or perhaps vesicular staining. This may be due to epitopes that may or may not be accessible to the different antibodies or that the polyclonal antibody is detecting epitopes common to other members of the HSP70 family of proteins. The plasma membrane association of HSP70 is a property of many cancer cells or may also be a consequence of cellular stress, so we are preparing to study several alternative cell lines to further examine the C-peptide and HSP70 interaction. While the protective effects of C-peptide are likely to involve HSP70, the subcellular location and trafficking may differ in other cultured cells or *in vivo*. However, the information gained from the HEK293 cells may be useful for discovering HSP70 binding peptides similar to C-peptide and that have C-peptide-like activity and therapeutic potential.

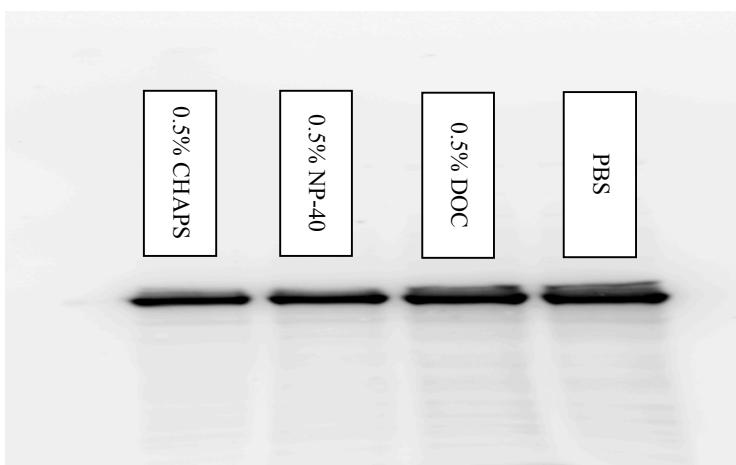
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We were able to prepare a sufficient quantity of the cross-linked molecule to be identified by mass spectroscopy. Our future work will focus on confirming the mass spectroscopy identification, exploring the biological and physiological significance of the identified protein, and investigating the presence and function of the identified protein in other cell lines that have shown physiological responses to C-peptide.

Our third quarterly scientific progress report for the third year of our project (03/28/13 – 06/27/13) described the following:

1. C-peptide binds specifically to HSP70.

We have shown previously with co-immunoprecipitation (Co-IP) assay that biotinylated C-peptides are able to pull down HSP70 proteins when supplemented to HEK293 cells cultured under serum free condition. To further demonstrate the interaction between HSP70 and C-peptide is specific, and to rule out the possibility that the HSP70 detected with Western blotting were pulled down nonspecifically by the streptavidin-coupled agarose beads, we repeated the co-IP experiments without photolink treatment.



Before eluting the proteins for Western blotting analysis, we subjected the agarose beads to **extra** washing steps with conditions that have previously been shown to be able to disrupt the non-specific binding between the pulled down proteins and the beads effectively (Figure 1 on the left). In comparison to PBS control, no major change of band intensity was observed under any these conditions, suggesting that HSP70 was pulled down by the beads via its interaction with the C-peptide. These results further validate the binding of C-peptide with HSP70 in HEK293 cells.

Figure 1. C-peptide binds specifically to HSP70. Detergent used in the extra washing step was listed on top of each lane. The eluted proteins were blotted with anti-HSP70 antibody.

2. C-peptide binding to HSP70 is independent of calcium influx.

It was shown previously that C-peptide is able to stimulate calcium (Ca^{2+}) influx in human umbilical vein endothelial cells (HUVEC) and renal tubular cells, thereby activating the Ca^{2+} -sensitive eNOS to protect these cells from hyperglycemia-induced apoptosis. To investigate whether the C-peptide/HSP70 interaction depends on Ca^{2+} levels, we added BAPTA AM, a cell-permeant Ca^{2+} chelator, to block any increase of intracellular Ca^{2+} concentration. As shown in Figure 2, selective depletion of intracellular Ca^{2+} did not affect the C-peptide binding to HSP70.

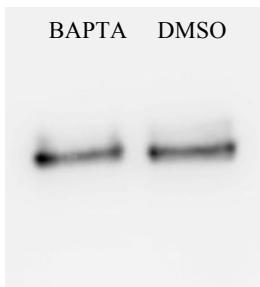


Figure 2. Western blot analysis of C-peptide/HSP70 binding in the presence of either Ca^{2+} chelator (BAPTA), or DMSO (as controls). The membrane is blotted with anti-HSP70 antibody.

3. C-peptide binding to HSP70 is independent of ATP regulation.

Central to the chaperon function of HSP70 is its capability to switch between the open and the closed conformation, which is powered through an allosteric mechanism via ATP binding and hydrolysis. In the presence of ATP, its polypeptide substrate binding domain (SBD) is in an open structure, which allows the access of the polypeptide to its binding groove; hydrolysis of the ATP to ADP will change the SBD to the closed conformation, locking the polypeptide within the groove and rendering the SBD inaccessible competing substrates.

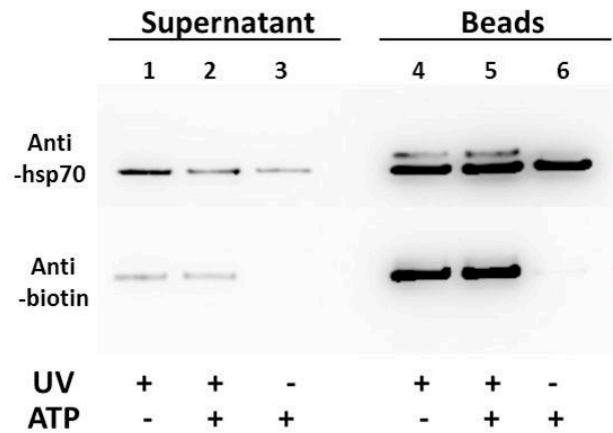


Figure 3. Western blot analysis of HSP70 co-immunoprecipitated with C-peptide in the presence or absence of ATP. Red arrow, HSP70 chemically cross-linked to biotinylated C-peptide; black arrow, HSP70 not cross-linked to C-peptide.

To examine whether the binding of C-peptide to HSP70 is also regulated by ATP, we co-immunoprecipitated HSP70 with biotinylated C-peptide as described before, with or without UV cross-linking of the photogenic leucine on the C-peptide. Before eluting the streptavidin-conjugated agarose beads with lysis buffer, we incubated the beads with HBSS in the presence or absence of ATP and collected the supernatants. If the C-peptide is lodged in the SBD domain of HSP70 and the binding is regulated by ATP, incubation with ATP will maintain the SBD at “open” conformation and release the C-peptide. As a result, uncross-linked HSP70 molecules will be eluted from the beads. In contrast, HSP70 cross-linked to C-peptide will be retained in the agarose bead. As shown in Figure 3 (lanes 3 and 6), HSP70 molecules are predominantly present on the beads, even in the presence of ATP, suggesting that maintaining the SBD in the “open” conformation will not affect the interaction between C-peptide and the HSP70.

Interestingly, among all the HSP70 molecules pulled down by the biotinylated C-peptides, only a percentage of HSP70 molecules are cross-linked (bands indicated by red arrows in Figure 3). One explanation is that UV-mediated cross-linking is not 100% efficient, and the majority of the HSP70 molecules co-immunoprecipitated with C-peptides is not chemically linked (bands indicated by black arrows in Figure 3). Alternatively, there might be multiple, non-mutually exclusive C-peptide binding sites on a single HSP70 molecule: the canonical polypeptide binding site in the SBD domain and the C-peptide specific binding site(s). Presumably, binding of C-peptide with the SBD domain is similar to other polypeptides and is regulated by ATP, whereas the putative non-canonical binding site is specific to C-peptide and is indispensable of ATP. Nevertheless, under both conditions (UV treated or untreated), the non-cross-linked HSP70 molecules are predominantly retained on the beads, suggesting that C-peptide binding to HSP70 is largely independent of ATP regulation.

4. Temperature control of C-peptide binding to HSP70.

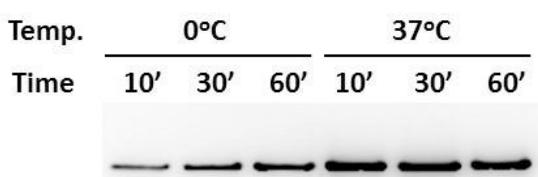


Figure 4. Western blotting analysis of HSP70 co-immunoprecipitated with C-peptide under different incubation conditions.

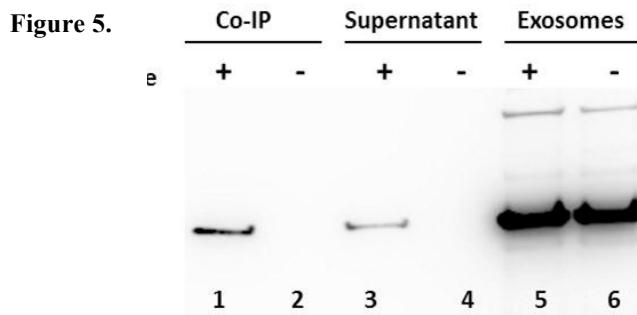
Most of the previously studies on C-peptide binding proteins were performed at 4°C. To further understand the thermodynamics of C-peptide binding to HSP70, and to examine whether such interaction occurs under normal physiological conditions, we incubated HEK293 cells with 50nM biotinylated C-peptides at 37°C from 10 to 60 minutes, and examined the amount of HSP70 co-immunoprecipitated (Figure 4). As shown, more HSP70 molecules were pulled down when the incubation time was extended from 10' to 60' at 0°C. In contrast, more HSP70 molecules were pulled down at 37°C, and no major change of HSP70 was observed when the incubation time was extended from 10' to 60'. These results suggest that the binding of C-peptide to HSP70 occurs rapidly at 37°C.

5. Absence of C-peptide/HSP70 complex in HSP70-containing exosomes.

Acting primarily as a cytoplasmic chaperone to facilitate proper folding of naïve proteins and to target aberrantly folded or damaged proteins for degradation, HSP70 is also actively released from the cells in exosomes under various stimulation conditions, notably heat-shock and stress (e.g. infections or tumor

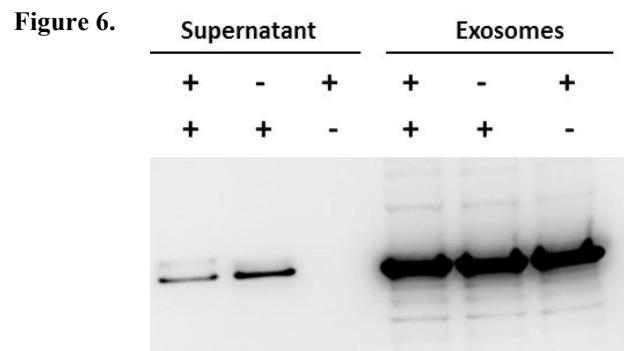
burden). Studies have shown that exosomes with surface bound HSP70, but not their negative counterparts, have the capacity to stimulate the innate immune response and exert immunomodulation. Thus, depending on its intra- and/or extracellular localization, HSP70 can either mediate protection against stress-induced cell damage intracellularly or act as danger signals to stimulate the adaptive and innate immune system.

To investigate the effects of C-peptide on the exocytosis of HSP70 in exosomes, we incubated the HEK293 at 4°C for 45 minutes, with C-peptides or without C-peptides. The incubation media were harvested, and were subjected to ultra-speed centrifugation at 45,000rpm for two hours. The amounts of HSP70 in the exosomes were assessed with Western blot analysis, with anti-HSP70 antibody (Figure 5, lanes 5 and 6). No major band intensity difference was observed in the presence or absence of C-peptide, suggesting that C-peptide does not affect exocytotic release of HSP70 via the exosome route.

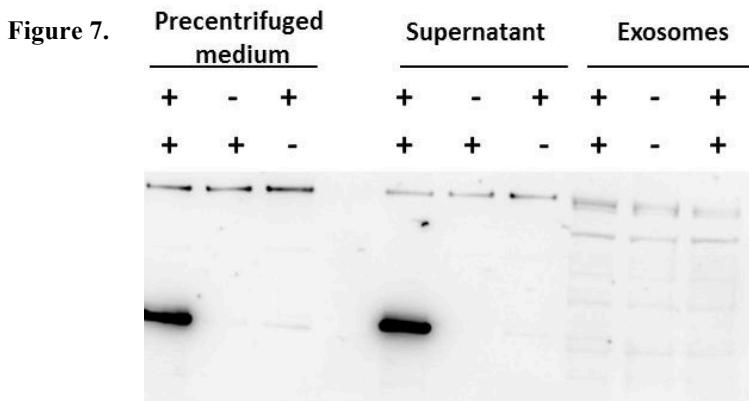


from the original incubation medium (Figure 5, lane 1), a slight decrease of band intensity was observed, suggesting that a substantial portion of the C-peptide/HSP70 complex was not present in the exosomes.

As shown in Figure 3 that only a percentage of the C-peptide/HSP70 complex is chemically cross-linked. To investigate the distribution of these cross-linked C-peptide/HSP70 complexes, we treated the HEK293 cells with UV in the presence or absence of C-peptide, and performed the above experiments. As shown in Figure 6, both the chemically linked form of the C-peptide/HSP70 complex (red arrow in Figure 6) and the uncross-linked form (black arrow in Figure 6) are present in the supernatant. In contrast, no chemically linked form is detected in the exosome samples (Figure 6, right panels). To further demonstrate that the chemically linked form is predominantly present in the supernatant, we repeated the experiment and probed



the Western blot with anti-biotin antibody, which detects only the chemically linked form of the C-peptide/HSP70 complex. Indeed, the biotinylated C-peptide/HSP70 complex is absent from the exosome lysates (Figure 7, right panel). Consistently, no signal intensity difference was observed between the HSP70 samples pulled down from the precentrifuge medium and the supernatant, proving further that the chemically linked C-peptide/HSP70 complex is present in the supernatant, but not in the exosomes.



In summary, we have further proved the bona fide binding of C-peptide to HSP70 in HEK293 cells. Our data suggest that there might exist two forms of C-peptide/HSP70 complex: C-peptide binding to the SBD of HSP70 which are regulated by ATP and can be chemically cross-linked with the photogenic leucine residue at position 24 of the C-peptide, and the non-canonical binding form. Moreover, our data clearly show that the chemically linked form of C-

peptide/HSP70 complex is present in the supernatant, but not in the exosomes.

Milestones of the next quarter

In the next quarter, we will continue to investigate the potential function of the C-peptide/HSP70 complex. Specifically, we will address whether the similar complex are present in other cell types under stress conditions and whether there exist two different forms of C-peptide/HSP70 complexes in these cells. Moreover, we will examine the surface binding properties of the C-peptide/HSP70 complex.

In the fourth quarterly scientific progress report (06/28/13 - 09/27/13) of year 03, we now report on our new and year 03 cumulative results.

Our studies have been conducted primarily with HEK293 cells that express abundant amounts of HSP70. We have examined the abundance of HSP70 in other cell lines by probing western blots of equal amounts of total soluble protein from a collection of cell lines. Figure 1 shows that the relative expression of HSP70 can be ranked in the order: HEK293 > K562 > Raji > Jurkat > HL60. No signal was detected for Daudi or CL01 cells. We have found that when compared with the HEK293 cells,

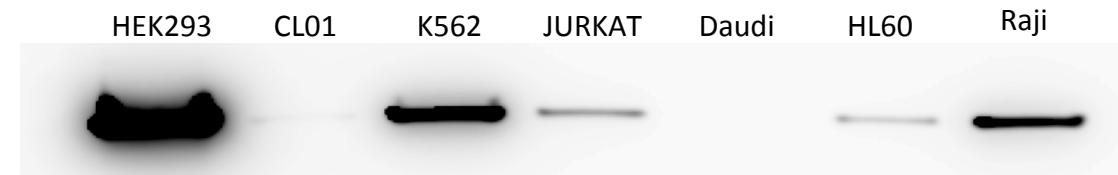


Figure 1. Abundance of HSP70 in cell lines. Cells from the indicated lines were treated with 1% CHAPS for 10 minutes, then centrifuged at 1,000 x g. The supernatant was filtered (0.22 μ) and a western blot was prepared with 5 μ g of total protein from each cell line. The blot was probed with HSP70 Ab.

K562 cells release a relatively small amount of HSP70 to the extracellular fluid. Although the expression by K562 cells is significantly less than HEK293, we were able to detect evidence of crosslinking of our photo-activated C-peptide to HSP70 in soluble fractions of cells treated with 1% CHAPS, Figure 2. This experiment also shows that C-peptide is cross-linked to intracellular HSP70 when the two molecules are rendered accessible to one another because of cell lysis. In addition, when the cells are lysed after UV treatment, the C-peptide interaction is still able to pull down the released intracellular HSP70 without being cross-linked. We interpret these results to indicate that to the extent that C-peptide is taken up it is able to be bound and cross-linked to HSP70 and that the greater amount of C-peptide outside the cell can readily interact with HSP70 released by cell lysis. However these interactions may be facilitated by other factors presence in the cell lysate.

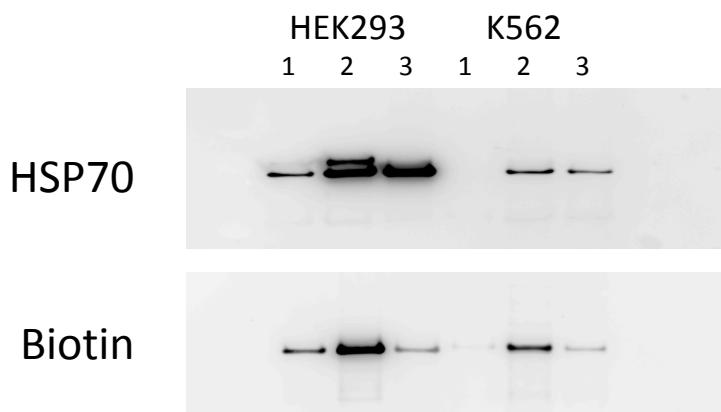


Figure 2. Comparison of HSP70 in HEK293 and K562 cells.
In the #1 lanes, HEK293 and K562 cells were treated with C-peptide, treated with UV light, and the extracellular fraction prepared in the usual way. In the #2 lanes, 1% CHAPS and protease inhibitor were added at the beginning, and in the #3 lanes, 1% CHAPS and protease inhibitor were added after UV treatment but before the centrifugation. Western blots were probed for HSP70 and biotin. The #1 lanes are lighter due to a shorter exposure time than our routine protocol because of the intensity of the other lanes. The cross-linked bands revealed via biotin correspond in size to the slower migrating band in the HSP70 blot, HEK293 lane 2.

We were concerned that protease could be released during cell lysis and could cleave our C-peptide and prevent the bead capture or otherwise interfere with the interpretation of our findings. In Figure 3, we show the results of a protease inhibitor cocktail on cross-linking and HSP70 pull-down in our basic experiments and when C-peptide is added to the cell-free supernatant. The western blot with HSP70 antibody shows a significantly reduced band corresponding to C-peptide cross-linked to HSP70 in the presence of the protease inhibitor. For the supernatant fractions, there is less HSP70 and no larger cross-linked bands. Although there was HSP70 present in the supernatant, and it was pulled down, the cross-linking appears to be much less effective. The cross-linked HSP70 bands are also revealed by another western blot detecting the presence of biotin and confirm the intracellular reduction of cross-linking by the protease inhibitor and the low degree of crosslinking in the supernatant. Based on these observations we speculate that both the C-peptide and protease inhibitors are taken up by the cells in a way that HSP70, C-peptide, and the protease inhibitors can interact with each other and with other factors involved in HSP70 peptide binding and release. While the possibility that an extracellular interaction depending on molecules at the external cell surface remains, many attempts to demonstrate HSP70 or HSP70-C-peptide exposed at the cell surface have not been conclusive. We are currently exploring the C-peptide cross-linking and pull down of lysed cell cell fractions to better demonstrate the HSP70 interactions and possibly other factors involved in the process.

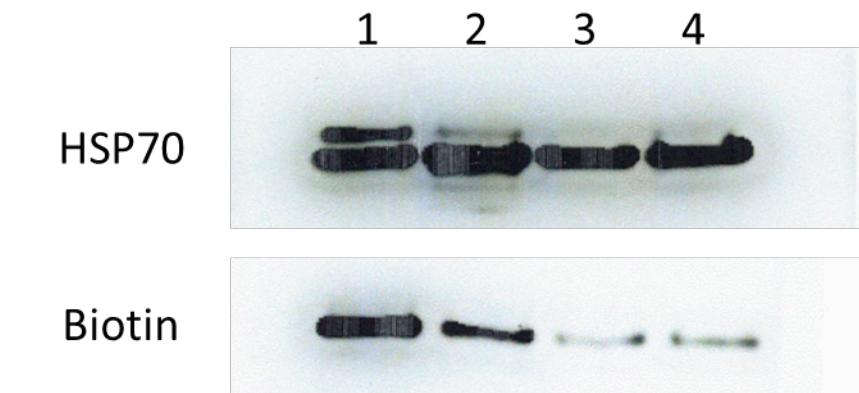


Figure 3. Effect of protease inhibitor on cross-linking.
HEK293 cells were treated with C-peptide or with C-peptide and protease inhibitor for 40 minutes, UV irradiated, and the supernatant prepared (lanes 1 and 2, respectively). The supernatant was first prepared and then C-peptide or C-peptide and protease inhibitor added followed by UV irradiation (lanes 3 and 4 respectively).

The variations of the sequence of C-peptides from species to species could affect their potential interactions with HSP70. We have had several different C-peptides synthesized to look for this possible difference in the interaction with HSP70. Table 1, shows the species and sequences we have studied.

Name	5' Tag	HA	C- Peptide Sequence
BHACPL24	Biotin	GYPYDVPDYASLR	EAEDLQVGQVELGGGGAGSLQP(Photo-L)ALEGSLQ
Sheep	Biotin-G		EVEGPQVGCALEAGGGAGG-----LEGPPQ
Mouse2	Biotin-G		EVEDPQVAQLELGGGGAGDLQT-----L-ALEVAQQ
Pig	Biotin-G		EAENPQAGAVELGGGLGG--LQA-----L-ALEGPPQ
Reverse	Biotin-G		QLSGELALPQLSGAGPGGGLEVQGVQLDEAE

Table 1. Biotinylated C-peptides from different species. C-peptides were synthesized with an added N-terminal biotin and glycine. Except for the reverse C-peptide, dashes are added to give an approximate alignment. BHACPL24 contains a photolinkable leucine at position 24 of the Human C-peptide sequence.

Although we intended to study these interactions using biotin to capture the HSP70-C-peptide complexes, background signals from endogenously biotinylated proteins made the results difficult to interpret. We then studied the ability of these C-peptides to block the capture and cross-linking mediated

by one of our HA tagged C-peptides. Figure 4 shows that the BHACPL24 peptide interaction was reduced by the presence of native C-peptide or pig C-peptide > mouse2 or reverse C-peptide > sheep C-peptide. The same order obtained by the capture and biotin detection of C-peptide cross-linked to HSP70.

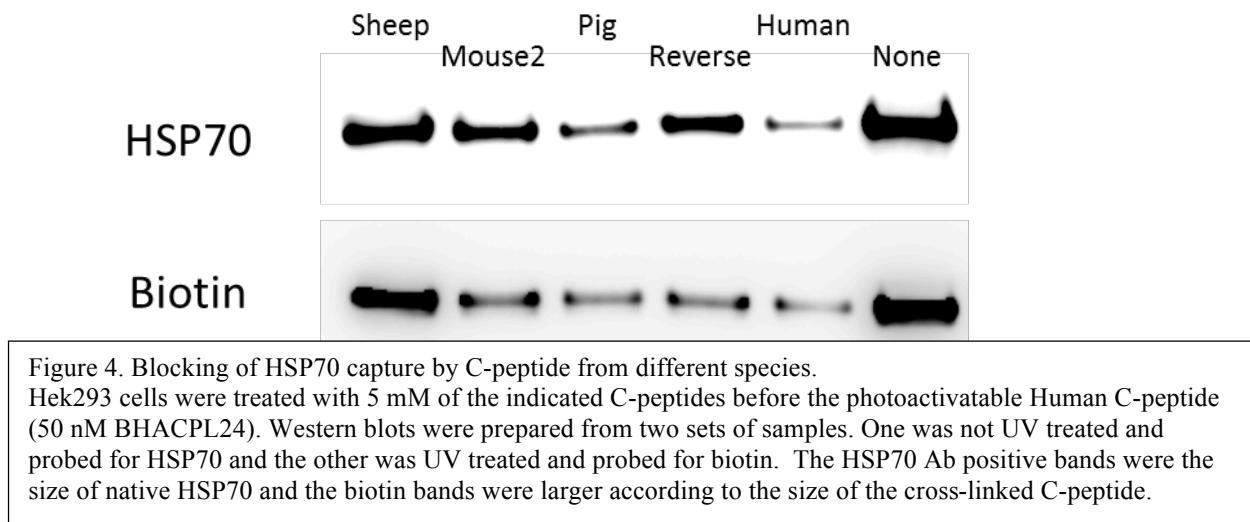


Figure 4. Blocking of HSP70 capture by C-peptide from different species.

Hek293 cells were treated with 5 mM of the indicated C-peptides before the photoactivatable Human C-peptide (50 nM BHACPL24). Western blots were prepared from two sets of samples. One was not UV treated and probed for HSP70 and the other was UV treated and probed for biotin. The HSP70 Ab positive bands were the size of native HSP70 and the biotin bands were larger according to the size of the cross-linked C-peptide.

The similarity of the C-terminal pentapeptide of pig and sheep pentapeptide suggests that the difference in the blocking of pig and sheep C-peptide is not because of the differences with the human C-peptide pentapeptide. Thus, the preceding human sequence (LQPLA) that is deleted in the sheep sequence may be the significant difference that is relevant to the HSP70 interaction. In addition, the two amino (GS) acids which precede this sequence are absent in the pig sequence but do not seem to diminish blocking by the pig sequence. On the other hand the charged amino acid (D rather than S) preceding the mouse (LQTLA) is potentially the significant difference that diminishes the blocking of the human HSP70-C-peptide interaction by the mouse2 sequence. These results suggest that the seven amino acids that precede the C-terminal pentapeptide are those which are most important for the HSP70-C-peptide interaction. However, this does not exclude the involvement of flanking sequences. The blocking by the reverse C-peptide sequence was expected because this is a characteristic of peptides that interact with the peptide binding domain of HSP70 and is not typically seen in more stereo-specific ligand-receptor interactions.

In our previous quarter report, we had found that a 100,000 x g pellet of the extracellular fraction contained HSP70 most likely as part of exosomes. However, for the relatively large amount HSP70 that was analyzed by western blot, no cross-linking to C-peptide was seen. To determine if this pelleted HSP70 was a significant part of the extracellular fraction, we compared the whole extracellular fraction with the soluble portion obtained after 100,000 x g centrifugation. The results in Figure 5 show that by western blot the amounts of HSP70 in the whole or soluble fraction were nearly equal and that treatment with C-peptide did not visibly alter the distribution of HSP70. This indicates that the HSP70 that is pelleted and presumed to be associated with exosomes is a small fraction of the extracellular HSP70 and by our previous findings it does not include the fraction of the extracellular HSP70 that is cross-linked to C-peptide.

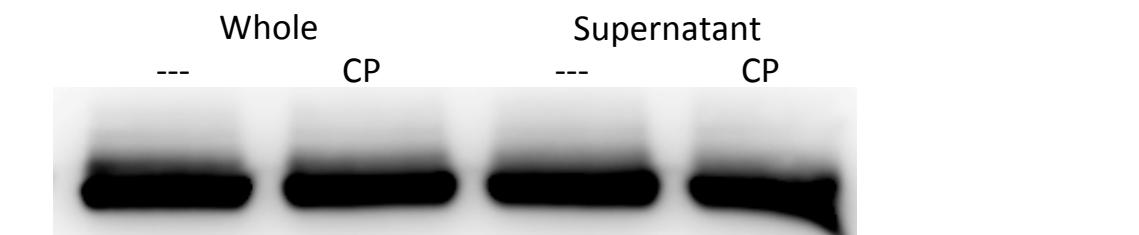


Figure 5. Most extracellular HSP70 is not removed by ultracentrifugation.

The extracellular fraction was prepared from HEK293 cells treated with or without C-peptide #21 as in our routine experiments. A portion of the fractions were saved and the remainder was centrifuged at 100,000 x g for 2 hours. The supernatants were compared with the whole extracellular fraction by western blotting using HSP70 Ab.

KEY RESEARCH ACCOMPLISHMENTS:

- * Identified HSP70 as a C-peptide binding protein
- * Tagged C-peptide readily captures HSP70 even without cross-linking.
- * Human C-peptide and several related molecules compete in our binding assay.
- * Provided evidence that the C-peptide binding involves the HSP70 peptide-binding site.
- * The C-peptide interaction involves amino acids which precede the C-terminal pentapeptide

REPORTABLE OUTCOMES: Manuscripts (1 publication)

Haidet J, Cifarelli V, Trucco M, Luppi P: C-peptide reduces pro-inflammatory cytokine secretion in LPS-stimulated U937 monocytes in condition of hyperglycemia. *Inflammation Research* 6:27, 2012.

Cifarelli V, Lee S, Kim DH, Zhang T, Kamagate A, Slusher S, Bertera S, Luppi P, Trucco M, Dong HH: FOXO1 mediates the autocrine effect of endothelin-1 on endothelial cell survival. *Mol Endocrinol*. 26:1213, 2012.

Haidet J, Cifarelli V, Geng X, **Trucco M**, Luppi P: C-peptide: A new molecule with anti-inflammatory properties. In: Sima A.A.F (Editor), **Diabetes & C-Peptide: Scientific and Clinical Aspects, Contemporary Diabetes**, Springer, pp. 111-127, 2012.

CONCLUSION:

To summarize our results for year 3, we have found a definitive interaction with a molecule that has been identified to be HSP70. Native-sequence C-peptide is able to block this process, and even the binding of a non-cross-linked C-peptide can be used to capture HSP70. So far our findings suggest that HSP70 is interacting with C-peptide just as it does with other peptides. Although we have not yet been able to directly demonstrate a role for this HSP70-C-peptide interaction in mediating the effects attributed to C-peptide, we note that many of the reported effects of C-peptide overlap with effects reported for HSP70 especially those on inflammatory pathways.

Importantly, in all of our studies, we have not found any other cross-linked molecule that could be a more dedicated receptor of C-peptide. Such an elusive receptor might still exist, but our findings suggest that HSP70 may still have a significant role in the intracellular and extracellular trafficking of C-peptide, and possibly in signaling processes. The HSP70 interaction with C-peptide may also modify the availability of C-peptide to other receptors. Also, cells that increase HSP70 expression in response to stress may respond differently to C-peptide than in their unstressed state.

The So What Section:

What is the implication of this research?

The World Health Organization estimated that 5–10% of all diabetes cases are T1D, i.e., 11–22 million worldwide and ~1 million only in US, where, with an incidence that increases by about 3% per year, it was the primary cause of diabetes in children less than 10 years of age. Diabetes affects 16 million Americans and roughly 5-15% of all cases of diabetes are T1D. It is the most common metabolic disease of childhood, and physicians diagnose approximately 10,000 new cases every year. T1D is associated with a high morbidity and premature mortality

due to complications, like cardiovascular diseases, nephropathy, retinopathy and neuropathy. T1D was estimated to cause \$10.5 billion in annual medical costs and an additional \$4.4 billion in indirect cost. \$1 of every \$7 dollars of US health is spent for medical care of diabetes without considering the loss of productivity. C-peptide administered regularly in physiological quantities might be an additive to insulin able to reduce those complications.

What are the military significance and public purpose of this research?

As the military is a reflection of the U.S. population, improved understanding of the underlying etio-pathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patients' well being.